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Cloned cDNA and Antibody for an Ovarian Cortical Granule Polypeptide of the Shrimp *Penaeus vannamei*

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Abstract. A cloned cDNA was generated to a transcript for a major ovarian polypeptide (200 kDa) of the South American white shrimp, *Penaeus vannamei*. The cloned cDNA hybridized to a single transcript in ovaries but not to RNA from the hepatopancreas or muscle. For immunodetection and quantitation, a monospecific polyclonal antibody was raised against the cDNA translation product expressed in bacteria. The antibody was used to show that the 200 kDa ovarian polypeptide accumulated in cortical granules during ovarian development to comprise ~11% of the total ovarian protein and disappeared during early embryonic development. These studies begin to explain a gene-product relationship essential for reproduction in *P. vannamei*.

Introduction

Cortical granules are large and abundant in the mature oocytes of penaeid shrimp (Duronslet *et al.*, 1975; Clark and Lynn, 1977; Clark *et al.*, 1980; Anderson *et al.*, 1984; Tom *et al.*, 1987; Bell and Lightner, 1988; Tan-Fermin and Pudadera, 1989). However, despite the prominence of these organelles, shrimp cortical granule synthesis and function have received little attention. Histological studies have shown that cortical granules contain glycoproteins and lack lipids (Tan-Fermin and Pudadera, 1989), but the precise nature of the protein moieties or other potential components has not been determined.

In the present studies we have quantified cortical granule size and abundance in mature oocytes of the South American white shrimp, *Penaeus vannamei;* isolated a cloned cDNA for a major cortical granule polypeptide; and, after making a polyclonal antibody against the genetically engineered cDNA translation product, quantified the amount of that cortical granule polypeptide during ovarian and very early embryonic development. The cloned cDNA and its corresponding antibody are sensitive probes for studies on regulation of a gene that is abundantly expressed during ovarian development in *P. vannamei*.

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Materials and Methods

Animals

Broodstock female *P. vannamei* were obtained from Laguna Madre Shrimp Farm (Los Fresnos, Texas), and Sea Critters Inc. (Tavernier, Florida). Shrimp were maintained in 2000-l circular tanks [biologically filtered, aerated, artificial seawater (35‰)] at 28°C under a 16L:8D (dim light) photoperiod, and fed squid and oysters twice daily. Under these conditions, unilateral eyestalk ablation induces ovarian development within two weeks (Rankin *et al.*, 1989). Shrimp spawn samples were obtained from Granada Genetics Inc. (College Station, Texas).

Ovarian cDNA library construction

Total RNA was isolated (Chirgwin *et al.*, 1979) from ovaries in mid-development (~150 μm oocyte diameter), and poly(A)$^+$RNA was selected by two rounds of oligodeoxythymidylate-cellulose chromatography (Aviv and Leder, 1972). Double-strand cDNA was prepared according to Gubler and Hoffman (1983), made blunt-ended with T4 DNA polymerase, and ligated to *Eco*RI/*Not*I linker-adaptors (Invitrogen). cDNAs ≥ 1.5 kb (~2 μg) were isolated by electrophoresis in agarose and glass powder adsorption (Vogelstein and Gillespie, 1979). The cDNAs were ligated to DNA of bacteriophage λgt11

(Young and Davis, 1983), and packaged with a commercial extract (Gigapack Plus, Stratagene Cloning Systems). Propagation on *Escherichia coli* Y1088 gave a library of 1.3×10^6 primary recombinants.

Isolation of a cloned cDNA representing an abundant ovarian transcript

Two mixed cDNA probes were used to isolate an ovarian cDNA representing a highly expressed transcript for a high molecular weight translation product. The first probe was ^{32}P-cDNA (Schleif and Wensink, 1981) prepared from total ovarian poly(A)$^+$RNA. The second cDNA probe was ^{32}P-cDNA prepared from 5–7 kb poly (A)$^+$RNA isolated from low-melting-temperature agarose gel. Duplicate plaque lifts on nylon filters (NEN Research Products) were hybridized with the two probes in 50% formamide, 4 X SSPE (20 X is 3.6 *M* NaCl, 0.2 *M* phosphate buffer pH 7.4, 20 m*M* EDTA), 2% SDS, 0.5% nonfat dry milk at 42°C overnight, and washes were in 15 m*M* NaCl, 10 m*M* Tris-HCl pH 7.4, 1 m*M* EDTA, 1% SDS at 60°C. Several recombinant bacteriophage hybridized strongly with both cDNA probes. A 3 kb cDNA insert from one of these recombinants was used for the experiments described below.

Production of a cDNA-derived antibody

The 3 kb ovarian cDNA (see above) was subcloned into the β-galactosidase C-terminal coding region of the plasmid expression vector pUR 291 (Ruther and Müller-Hill, 1983) for *E. coli* JM101 transformation. A fusion polypeptide (part bacterial β-galactosidase and part shrimp cDNA translation product) was induced by addition of isopropyl-β-D-thiogalactopyranoside to cell cultures in log-phase. Cellular polypeptides were separated by SDS-PAGE (Laemmli, 1970). The fusion polypeptide (205 kDa) was visualized with KCl, electroeluted (Andrews, 1986), and quantified according to Lowry *et al.* (1951). A polyclonal antibody to the gel-purified fusion polypeptide was raised in 3-month-old New Zealand white rabbits. Freund's complete adjuvant was included in the primary injection of ~600 μg protein, and the non-pyogenic adjuvant T1501 (gift from Dr. L. F. Woodard, Dept. Vet. Sciences, University of Wyoming) was used in booster injections (~150 μg protein). Rabbit serum was diluted with 15 m*M* phosphate buffer pH 6.4, and the IgG fraction isolated with a prepacked diethylaminoethyl-cellulose ion exchange column (Pharmacia LKB Biotechnology) according to the supplier's instructions. The IgG was lyophilized and stored at −80°C until use.

Ovarian transcript characterization

Total RNA was isolated from selected shrimp tissues, denatured with MeHgOH (Bailey and Davidson, 1976), and separated by electrophoresis in 1.2% agarose gel. RNA was transferred to a nylon filter (Zeta-Probe, Bio-Rad Laboratories) and hybridized with the nick-translated (Rigby *et al.*, 1977) cloned ovarian cDNA under conditions described above for cDNA isolation. Hybridization was visualized with autoradiography.

Immunoblot analysis

Polypeptides were separated by SDS-PAGE (7.5% gel) and transferred to nitrocellulose using a semi-dry electroblotting apparatus (American Bionetics) according to the manufacturer's recommendations. Polypeptides reacting with the fusion polypeptide antibody were visualized with an alkaline phosphatase detection system (Bio-Rad).

Histochemistry

Ovary fragments were fixed in 4% formaldehyde in Ott's artificial seawater (Spotte, 1979), embedded in paraffin, and sectioned (5 μm) (Sheehan and Hrapchak, 1980). Immunoreactivity in sections was located with the Bio-Rad alkaline phosphatase detection system. Control samples were incubated with IgG from uninjected rabbits. Determination of oocyte and cortical granule parameters were made on sections of 62 cells with nuclear diameter > 44 μm, using a light microscope (160×) with ocular micrometer. Analysis of cell surface sections (n = 42) was used to determine the number of cortical granules per cell.

Enzyme-linked immunosorbent assay (ELISA)

Samples in 60 m*M* carbonate buffer pH 9.6 were adsorbed to Immulon 2 Plates (Dynatech) at 4°C overnight. The plates were washed with 0.33 *M* phosphate-buffered saline pH 7.2 (PBS), and PBS adjusted to contain 0.001% Tween-20. Wells were blocked with ovalbumin (5% in carbonate buffer), washed, and incubated in PBS with rabbit IgG (see below). Immunoreactive protein was assessed at 405 nm with a peroxidase-linked antibody to rabbit IgG. For quantitation, varying quantities of fusion protein were run as standards against which the crude tissue extracts were compared. This quantitation required two preliminary experiments: (1) adsorption of the antibody with varying concentrations of a pUR 291/*E. coli* lysate. This experiment determined the concentration of cell lysate necessary to effectively remove the antibodies directed toward contaminating *E. coli* proteins. The most abundant contaminant was the 115 kDa β-galactosidase portion of the fusion protein. Adsorption of the antibody was essential to ensure that only the epitopes common to both the fusion protein and the shrimp ovary polypeptide were detected in the ELISA. Only appropriately adsorbed antibodies were used in subsequent

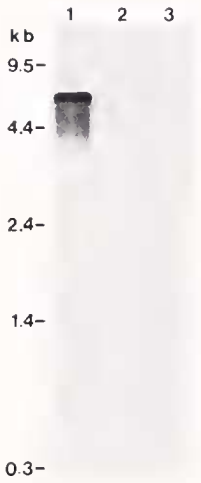

Figure 1. Total RNA samples (5 μg/lane) from mid-reproductive cycle *Penaeus vannamei* tissues were denatured, separated by electrophoresis in agarose, transferred to nylon membrane, and hybridized with the cloned 3 kb ovarian cDNA. Lane 1, ovary; lane 2, hepatopancreas; lane 3, muscle. RNA size markers are indicated at left.

experiments. (2) Incubation of extracts of various concentrations of pre-vitellogenic ovaries and nearly mature ovaries with various concentrations of fusion protein. This experiment demonstrated that the fusion polypeptide and immunoreactive native ovarian protein were adsorbed to the incubation wells with equal efficiency. We assumed that the common epitopes on the two different molecules (*i.e.*, fusion protein and ovarian polypeptide) behaved identically in the ELISA.

Results

Transcript characterization

Northern hybridization was performed to determine (1) the size of the highly expressed transcript(s) represented by the cloned ovarian cDNA and (2) which tissues expressed transcripts for production of the major ovarian polypeptide. The cDNA hybridized with a single 6 kb transcript from a mid-cycle ovary (oocyte diameter ~ 150 μm) (Fig. 1). The cDNA did not hybridize with RNA from muscle or hepatopancreas of the same animal.

Genetically engineered fusion polypeptide

A genetically engineered polypeptide consisting of shrimp ovarian polypeptide and bacterial β-galactosidase was generated for subsequent immunological identification of the shrimp ovarian cDNA translation product. For generation of this fusion polypeptide, the 3 kb cloned cDNA representing the 6 kb ovarian transcript was inserted into plasmid pUR 291 (Ruther and Müller-Hill, 1983). This recombinant construct was used for high-level expression of the fusion polypeptide in bacteria. The fusion polypeptide consisted of ~115 kDa β-galactosidase combined with a ~90 kDa *P. vannamei* ovarian polypeptide sequence (Fig. 2).

Immunoblot analysis

A polyclonal antibody raised against the fusion polypeptide was used to determine the size and tissue distribution of the ovarian 6 kb mRNA translation product. Immunoblot analysis of tissues from a shrimp with mid-cycle ovaries showed strong reaction with a ~200 kDa ovarian polypeptide (Fig. 3). There were smaller, faintly reacting polypeptides in the ovary, and one small reacting polypeptide (~35 kDa) from the hepatopancreas. The antibody did not react with muscle or hemolymph samples.

Histochemical analysis

Localization of the ovarian polypeptide was accomplished by immunocytochemical analysis. Immunoreactivity was found in the cortical granules of the mature oocytes (Fig. 4). The cortical granules were prominent club-shaped corticular organelles, ~38 μm long, 12 μm in basal diameter, and ~1.4×10^3 μm^3 in volume calculated according to conical shape. Analysis of cell surface sections indicated that there were ~420 cortical gran-

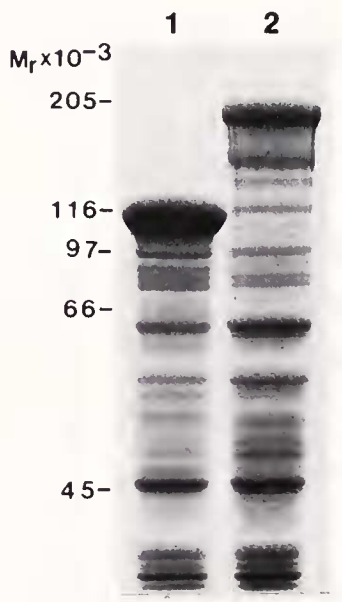

Figure 2. *E. coli* JM101 was transformed with plasmid pUR 291, and plasmid-encoded β-galactosidase was induced by addition of IPTG. Cell extracts were separated by SDS-PAGE (7.5%) and stained with Coomassie Blue R. Lane 1, native pUR 291; lane 2, recombinant pUR 291 containing the 3 kb ovarian cDNA. The heavy band at 116 kDa in lane 1 is unfused β-galactosidase. The band at 205 kDa in lane 2 is a fusion consisting of β-galactosidase linked to a polypeptide encoded by the ovarian cDNA.

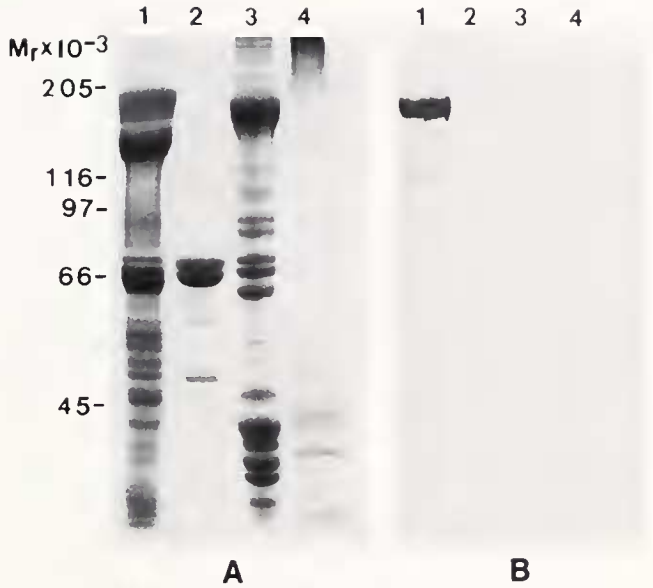

Figure 3. *Penaeus vannamei* polypeptides were separated by SDS-PAGE, and stained with Coomassie Blue R (Panel A), or blotted onto nitrocellulose and incubated with a polyclonal antibody to the genetically engineered fusion polypeptide (Panel B). Samples were from an animal with mid-cycle ovaries (oocyte diameter ~ 150 μm). Lane 1, ovary; 2, hemolymph; 3, muscle; 4, hepatopancreas. Positions of molecular weight markers are indicated at left.

ules/cell, comprising ~10% of the oocyte volume. These estimates are based on a cell diameter of ~226 μm (Table I). This cell diameter measurement was an underestimate of that in fresh tissue as a result of (a) cell shrinkage (from 320 μm, see below) during sample preparation and (b) imprecision in determining the cell center due to variations in cell morphology.

Developmental profile of the 200 kDa ovarian polypeptide

The contribution of the 200 kDa polypeptide to total protein was determined by ELISA during both ovarian development and embryonic development using the purified fusion protein as the standard. The 200 kDa polypeptide was not detected in a previtellogenic ovary (oocyte diameter = 0) (Fig. 5). It represented 4–5% of the total ovarian protein at oocyte diameter 200–240 μm and ~11% of the protein in a fully developed ovary (320 μm). In newly spawned eggs, the 200 kDa polypeptide comprised 3–4% of the protein. By 2 h after the spawn, the 200 kDa polypeptide had declined to <1% and was not detected in ≥4 h spawn. By 6 h after spawning, the first antennal, second antennal, and mandibular primordia were visible, and hatching occurred 16–18 h after spawning.

Discussion

Cortical granules are found widely in vertebrates and invertebrates; however, the size, abundance, and composition of these granules varies among species (see Guraya, 1982, for review). As shown by light and electron microscopy, cortical granules in mature ovaries of penaeid shrimp are large and plentiful (Duronslet *et al.*, 1975; Clark and Lynn, 1977; Clark *et al.*, 1980; Anderson *et al.*, 1984; Tom *et al.*, 1987; Bell and Lightner, 1988; Tan-Fermin and Pudadera, 1989). We showed that, in *P. vannamei*, the cortical granules were club-shaped structures, about 38 μm long, and occupied about 10% of the volume in fixed, mature oocytes (Fig. 4, Table I). In mature ovaries of *Penaeus aztecus*, the somewhat smaller but

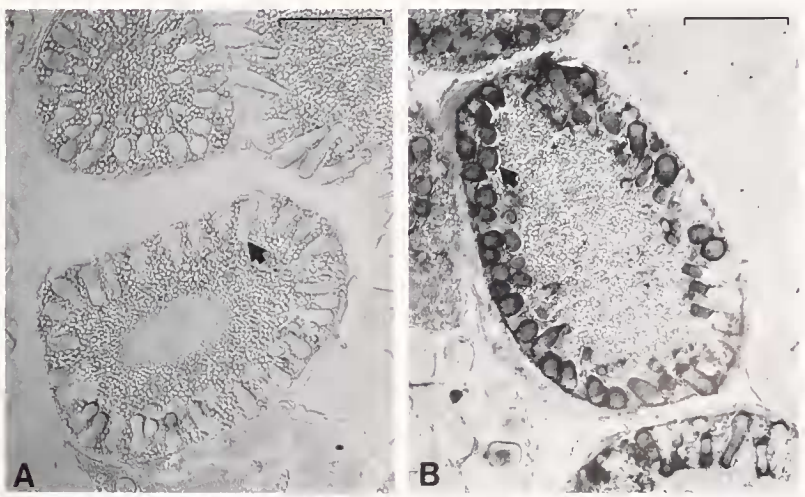

Figure 4. Localization of the 200 kDa ovarian polypeptide using immunocytochemistry. Ovarian sections (5 μm) were incubated with (Panel A) preimmune rabbit IgG and (Panel B) IgG from the rabbit inoculated with the gel-purified fusion polypeptide (see Fig. 2). Immunoreaction was visualized with an alkaline phosphatase-linked second antibody. Arrows indicate cortical granules. Scale bars = 100 μm.

Table I

Estimates of oocyte and cortical granule parameters in mature ovaries of* Penaeus vannamei

Parameter	Size ± SD
Oocyte diameter	226 ± 16 μm
Oocyte surface area	1.6×10^5 μm^2
Oocyte volume	6.0×10^6 μm^3
Cortical granule length	37.8 ± 4.3 μm
Cortical granule diameter (a)	12.0 ± 1.4 μm
Cortical granule diameter (b)	7.5 ± 1.2 μm
Vol. of cortical granules	1.4×10^3 μm^3
No. cortical granules/cell	420 ± 70
Vol. cortical granules/cell	5.9×10^5 μm^3
Vol. cortical granules/cell vol (%)	~10%

* Measurements were taken from 5 μm sections after fixation, embedding, deparafinization, and rehydration. Sections of 62 cells with nuclear diameter > 44 μm were measured for the various parameters. To determine cortical granule dimensions, three granules per cell section were measured for length, apical (a), and basal diameter (b).

more numerous cortical granules occupy about 12% of the oocyte volume (Clark *et al.*, 1980). Histological analysis shows that cortical granules of *Penaeus monodon* stain positively with alcian blue-periodic acid Schiff, indicating glycoprotein components, and negatively with Sudan black, suggesting an absence of lipids (Tan-Fermin and Pudadera, 1989).

The studies described in this paper resulted in a cloned cDNA and antibody for one highly expressed cortical granule polypeptide of the shrimp, *P. vannamei*. Using these probes, regulation of shrimp reproduction at the level of a single gene can be examined for the first time. Bacterial expression of the cloned cDNA (Fig. 2) allowed production of a monospecific antibody directed against the translation product. Immunoblot analysis showed that the major immunoreactive polypeptide (200 kDa) and several faintly reacting, smaller polypeptides were either immunologically related products of more than one gene, or, alternatively, natural or artifactual cleavage products of the 200 kDa polypeptide. We regard the latter possibilities as the more likely since the ovarian cDNA hybridized to only a single ovary-specific transcript (Fig. 1). Most likely, the immunoreactive polypeptide in the hepatopancreas originated in the ovary and, through leakage and absorption, was detected subsequently in the hepatopancreas.

We demonstrated by immunocytochemical analysis that the 200 kDa polypeptide was localized in cortical granules (Fig. 4) and that it accrued from virtually undetectable levels in the immature ovary up to ~11% of the total protein in the mature ovary of *P. vannamei* (Fig. 5). Because it is strikingly abundant (Fig. 5) and relatively insoluble (Rankin *et al.*, 1989), we speculate that the 200 kDa polypeptide contributes to the structural integrity of the cortical granules. This polypeptide is similar in size to a 180–193 kDa polypeptide found in cortical granules of the sea urchin *Strongylocentrotus purpuratus* (Kopf *et al.*, 1982; Villacorta-Moeller and Carroll, 1982). Whether sea urchin cortical granule polypeptides are immunologically related to those of *P. vannamei* remains to be determined. Indeed, determination of similarities and differences between cortical granules within single oocytes is an area of active research. Among the crustaceans, ultrastructural observations indicated two types of cortical granules in the horseshoe crab, *Limulus polyphemus* (Bannon and Brown, 1980); two in the fairy shrimp *Tanymastix* (Garreau de Loubresse, 1974); and four in the lobsters *Homarus americanus* and *H. gammarus* (Talbot and Goudeau, 1988). In the sea urchin *S. purpuratus*, immunological analysis shows a marked heterogeneity of distribution for a cortical granule polypeptide, suggesting differences between cortical granules in that species (Anstrom *et al.*, 1988).

Cortical granule contents are thought to originate in the oocytes (see Guraya, 1982, for review). For example, in crustaceans, ultrastructural evidence indicates that at least one type of cortical granule, now termed "ring-shaped inclusions," of the crab *Carcinus maenas* (Goudeau, 1984) and the lobster *Homarus* (Talbot and Goudeau, 1988) are products of oocytic machinery. We are the first to apply molecular genetic analysis to demonstrate an ovarian origin for a major cortical granule polypeptide (Figs. 1–3). It is likely that this polypeptide is synthesized in the oocytes; however, this remains to be determined by *in situ* hybridization.

The 200 kDa cortical granule polypeptide accumu-

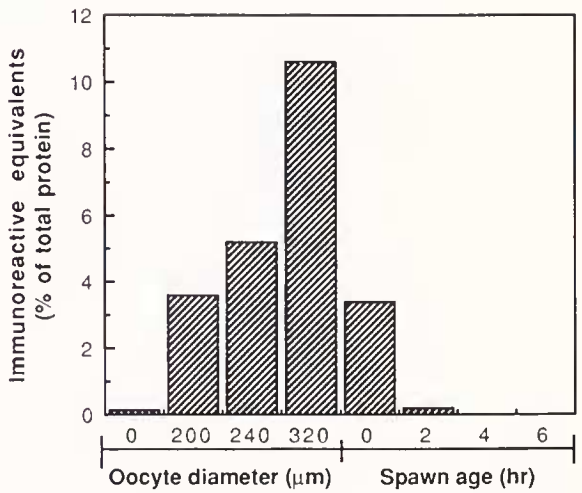

Figure 5. Enzyme-linked immunosorbent assay of the 200 kDa ovarian polypeptide, in total protein from developing ovaries and spawn. The purified fusion polypeptide (Fig. 2) was the antigen and protein standard. The fusion polypeptide antibody was preadsorbed to an *E. coli* lysate.

lated during ovarian development, from a virtually undetectable level in a previtellogenic ovary to ~11% of the total protein in a fully developed ovary. Within a few hours of spawning, the 200 kDa polypeptide had disappeared (Fig. 5), presumably through exocytosis during the cortical reaction (see Clark *et al.*, 1980). The cDNA and antibody that we have generated for this polypeptide can now be used to deduce the primary structure of a major component of the cortical granules and determine its site of synthesis precisely; to characterize the maturation of cortical granules; and to investigate the regulation of synthesis of this major ovarian component at the pretranslational and translational levels.

Acknowledgments

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